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13. SUPPLEMENTARY NOTES

14. ABSTRACT Fragile X syndrome (FXS) is the most common heritable form of intellectual disability, the most common single-gene form of autism, and a relatively common cause of epilepsy. The syndrome is caused by partial or complete silencing of the fragile X (*FMR1*) gene when a CGG-repeat element in the gene expands to more than 200 repeats, leading in turn to loss of the *FMR1* protein (FMRP). The protein is important for brain development, and its loss is accompanied by both intellectual and behavioral disability. Accordingly, the central objective of the proposed research is identification of therapeutic agents that stimulate production of FMRP from residual *FMR1* messenger (m)RNA in neurons, thereby reversing the effects of decreased gene activity. Our approach is twofold: (i) to block repressive interactions between microRNAs and the 3′ non-coding portion of the *FMR1* message, thereby leading to increased protein levels; (ii) to screen a large library (~20,000 compounds) of small molecules, each having the potential for crossing the blood-brain-barrier, for those with the ability to increase FMRP levels. Increasing the expression of FMRP holds the potential to correct ALL of the clinical domains of fragile X syndrome, including epilepsy-like activity observed for both those with FXS and carriers of smaller CGG-repeat expansions. Finally, posttraumatic stress disorder (PTSD) has been described in fragile X syndrome and in premutation carriers. Thus, the proposed studies may lead to treatments that reduce the PTSD risk as well, an issue of importance for the military personnel. Since the prevalence of fragile X syndrome is approximately 1 in 3,000 to 4,000 in the general population, nearly two-thousand children of service personnel are likely to have fragile X syndrome, with a much larger number (~7,500) of active military personnel being carriers of an expanded (premutation) form of the *FMR1* gene.

15. SUBJECT TERMS
Fragile X, autism, *FMR1*, FXTAS, CGG repeat, epilepsy, seizures, FMRP, PTSD, premutation, iPSC, progenitor, calcium regulation

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1. INTRODUCTION

Fragile X syndrome (FXS) is the most common heritable form of intellectual disability, the most common single-gene form of autism, and a relatively common cause of epilepsy. The syndrome is caused by partial or complete silencing of the fragile X (*FMR1*) gene when a CGG-repeat element in the gene expands to more than 200 repeats, leading in turn to loss of the *FMR1* protein (FMRP). The protein is important for brain development, and its loss is accompanied by both intellectual and behavioral disability. Accordingly, the central objective of the proposed research is identification of therapeutic agents that stimulate production of FMRP from residual *FMR1* messenger (m)RNA in neurons, thereby reversing the effects of decreased gene activity. Our approach is twofold: (i) to block repressive interactions between microRNAs and the 3′ non-coding portion of the *FMR1* message, thereby leading to increased protein levels; (ii) to screen a large library (~20,000 compounds) of small molecules, each having the potential for crossing the blood-brain-barrier, for those with the ability to increase FMRP levels. Increasing the expression of FMRP holds the potential to correct ALL of the clinical domains of fragile X syndrome, including epilepsy-like activity observed for both those with FXS and carriers of smaller CGG-repeat expansions. Finally, posttraumatic stress disorder (PTSD) has been described in fragile X syndrome and in premutation carriers. Thus, the proposed studies may lead to treatments that reduce the PTSD risk as well, an issue of importance for the military personnel. Since the prevalence of fragile X syndrome is approximately 1 in 3,000 to 4,000 in the general population, nearly two-thousand children of service personnel are likely to have fragile X syndrome, with a much larger number (~7,500) of active military personnel being carriers of an expanded (premutation) form of the *FMR1* gene.

2. KEYWORDS

Fragile X, autism, *FMR1*, FXTAS, CGG repeat, epilepsy, seizures, FMRP, PTSD, premutation, iPSC, progenitor, calcium regulation

3. OVERALL PROJECT SUMMARY

Task 1. *Identification of cis-elements for translational up-regulation within the FMR1 mRNA non-coding regions*

Summary of current objectives – The principal objective of Task 1 was to determine the extent to which blocking inhibitory interactions between microRNAs and the 3′UTR of the *FMR1* gene would lead to increased expression of the *FMR1* protein (FMRP). As noted in the previous Progress Report, we had largely completed this task. Based on the potentially much more useful results obtained under Task 2, we do not propose to extend these studies.

Summary of Results – We report no additional results beyond those described in detail in the previous progress report.

Progress – We have largely completed these studies.

Accomplishments/Discussion – In the last Progress Report (2014), we presented our results for the most promising blocking experiments. Following studies using a variety of oligomers to block the microRNA-mRNA interactions, and using various means for delivery, the best outcome was about 1.8-fold increase in eGFP reporter fluorescence; eGFP was the reporter for the plasmid construct with the *FMR1* 3′UTR, the target of the microRNAs. Although the increase was measurable, our experiments suggested that there would be two significant hurdles to the use of blocking oligomers as a treatment strategy. Specifically, the oligos are difficult to deliver to cells, and likely would be impractical as a therapeutic agent, given the modest increase observed in the cell-culture model. Therefore, in light of the discoveries made under Task 2, we do not plan any further work under Task 1.

Task 2. ***Screening of a specific library of ~20,000 small molecules for candidates capable of increasing FMRP and eGFP levels in transfected fibroblasts***

Summary of current objectives – The current objective of this task remains unchanged; namely, the screening of a panel of small chemical compounds to identify those compounds that increase the level of FMRP expression in a live-cell culture model.

Summary of Results – As discussed in our previous Progress Report, our original approach to the identification of candidate compounds was to use eGFP reporter plasmid-transfected fibroblasts, where the 5′ and 3′ UTRs of the *FMR1* gene were added to the eGFP coding region. However, one major shortcoming with this method was the fact that it was not measuring the level of endogenous FMRP, nor could this method be directly applied to alleles with expanded repeats. With our implementation of a FRET-based assay for FMRP, originally described by Schutzius et al. (2013) and modified by Cisbio (Cisbio US, Bedford, MA), these problems were eliminated, as is the need for a Western blot analysis as a second-stage analysis. The FRET-based approach has been fully implemented and validated during the current budget period, and has allowed us to analyze over 2,400 chemical compounds to date.

In the basic approach, we are now running eight 384-well plates per week (up from 4 plates per week in the initial assays), with each plate testing 80 compounds from the library (20,000* compounds, 2,400 tested to date) [*through cost savings; initially 18,000]. At the current rate, we expect to complete the primary screen within 4-5 months. As noted, one distinct advantage of this approach is that it allows us to use fibroblast lines with expanded CGG repeats (and low FMRP levels); thus, we can assess the potential for increased FMRP expression under conditions where its expression is known to be diminished. The FRET method, and the 384-well format, allows us to run multiple replicates of each compound as well as testing the effects of the carrier (dimethyl sulfoxide, DMSO) on FMRP expression.

In the first phase of our screening approach, we spent substantial time developing protocols for ensuring uniform response of the assay, which reflects consistent numbers of cells cultured in each of the 384 wells, and consistent delivery of lysing and detection solutions (**Figure 1**). We had initially thought to use liquid handlers for these tasks, but we could find no suitable automated handler that would allow us to preserve sterility for subsequent culture. Therefore, we decided to complete all tasks manually. At present, we have been able to process eight 384-well plates per week. Based on our analysis of multiple control samples across plates, we have chosen a value of 2.0 as the cutoff for “hit” compounds and p <0.001 from one-way ANOVA for each plate.

We have found that for most of the 2,400 test compounds analyzed to date, there is little variation in the relative FRET compared to the DMSO controls (**Figure 2**).

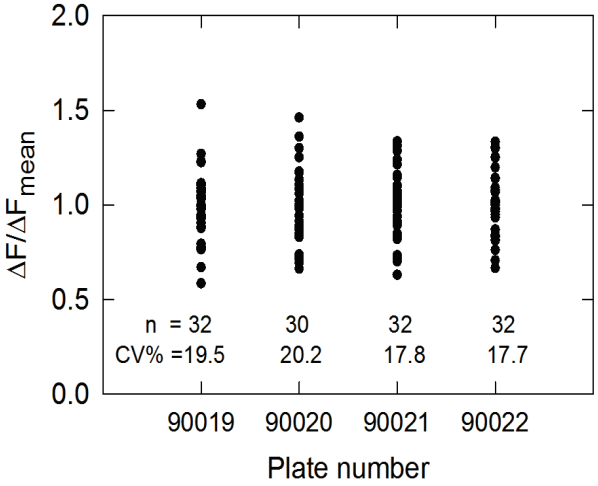

Figure 1. Example of the variation in FRET for the DMSO controls (1% DMSO final) for four plates. All compounds delivered to plated cells at a final concentration of 20 μM (1% final DMSO concentration). Each 384-well plate includes 30-32 wells (n) with DMSO without test cpds. CV, coefficient of variation, expressed as the mean of the control measures. A test value of 2.0 would be approximately 5σ above the DMSO mean.

This finding indicates that most compounds have little effect of FMRP expression, either because they intrinsically lack any effect on FMRP expression, or they may not be penetrating the cells in culture. For each plate of 80 compounds, we seed two 96-well plates at ~7500cells per 200µl RPMI and grow overnight, followed by serum starvation for 16 hr. The cells are then treated with 20µM of each compound (or DMSO only) containing 0.05% DMSO in RPMI and are incubated for a further 24 hr. The cells are then lysed, followed by FRET assay analysis.

In the primary screen (initial screen of all compounds), we transfer cultured fibroblasts to each well of a 384-well plate, followed by a 24 hr incubation period to allow the cells to adhere to the surface and undergo a period of stabilizing growth. We then add compounds (or DMSO) to each well, followed by another period of 24 hr. Following this second 24 hr period, the cells are lysed and the FRET analysis is performed. Of importance to these experiments, we are using a fibroblast line originally derived from an individual with 170 CGG repeats, whose FMRP level was reduced by approximately 3-fold from normal levels.

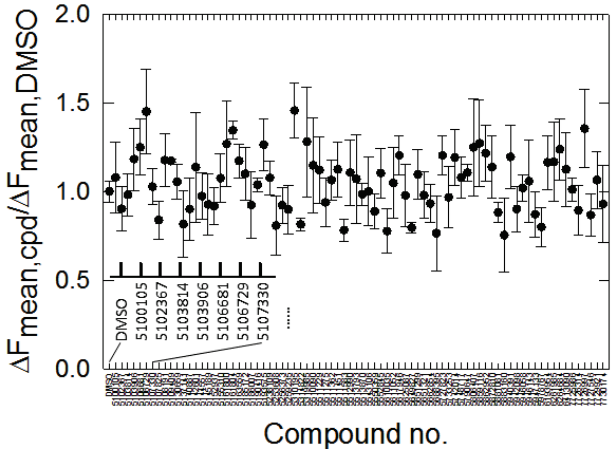

Figure 2. Example of 80 compounds (one sample plate; four replicate cultures per compound) where there is no significant effect on FMRP expression. (Inset) Blow-up of a portion of the X-axis showing compound number.

The truly exciting aspect of this work is that we have identified 31 "hits" on the primary screen that satisfy both the criterion that the mean elevation exceeds 2.0-fold and that $p < 0.001$, corrected for multiple outcomes through the plate-wise ANOVAs. The range for these primary hits (fold elevation of FMRP) extends from 2.05 to 12.6, with 15 hits in the 2.0–3.0 range, 9 hits in the 3.0–4.0 range, and 7 hits above 4.0 (see: **Figure 3**). We have begun to run secondary tests on the primary hits as we continue to perform the primary screen. Thus far, the secondary screens are bearing out the initial observations. To put these results in context, we have chosen a fibroblast line for which FMRP production is diminished three-fold, a reduction that is thought to be responsible for part of the clinical presentation in these individuals; sixteen of the hits exceed that three-fold reduction.

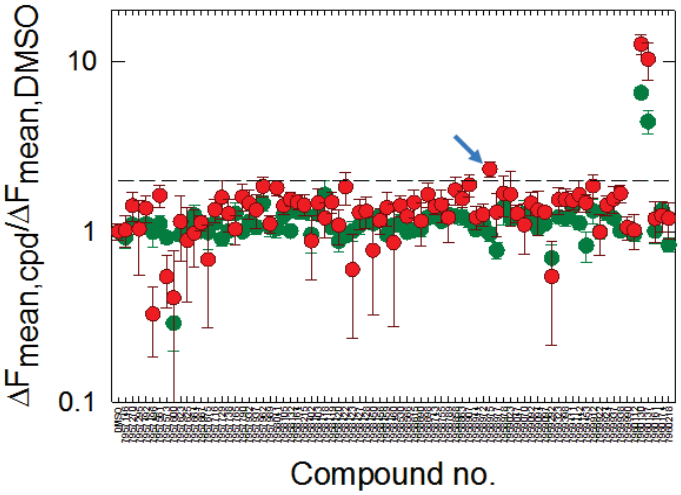

Figure 3. Semi-log plot demonstrating consistency of expression levels upon performing a repeat FRET analysis. Note that nearly all observations fall below a 2-fold increase (dashed line), except for the dramatically elevated level for a single sample (green); a repeat analysis yielded even higher values for that same sample, which is regarded as a "hit". One additional sample (arrow) slightly exceeds the 2-fold threshold for one analysis, but does not meet that threshold for the other analysis.

Progress – As noted in our Summary of Results, our major accomplishment thus far is the identification of approximately thirty molecules that, for a single dose of test compounds at 20 µM, lead to significant increases in FMRP expression. Since we are approximately 12% through the 20,000 compound set, we would project an additional 220 "hits" exceeding

2.0, with an estimated 58 of these exceeding a 4-fold increase. At this point, the only utility of this projection is to anticipate the amount of work necessary for secondary and tertiary screens.

We have begun to run secondary screens at this time – in parallel with the primary screen – in the hope that some of the hits may maintain their potential for use as a pharmaceutical agent.

Based on our current rate of screening, we should complete our primary screen in another 30–32 weeks (March of 2016), including time for secondary screening of the initial hits.

Accomplishments/Discussion – We have made two significant accomplishments related to this Task: (i) we have implemented and optimized a FRET-based assay that is now capable of screening our compound library for small molecules that result in an increase in FMRP in a live cell culture; (ii) we have identified a number of hit compounds that are capable of increasing FMRP levels in fibroblasts from an affected individual, where FMRP production is known to be impaired. Before we can comment further on the potential utility of any of the hit compounds, we will need to perform a number of additional studies to gain some idea of the basis for their action(s). Are they influencing transcription? Are they stimulating production of FMRP specifically or are they simply enhancing translation in general? What is their effective range and time-course of effect, etc? Further assessment of the hit compounds will take place over the next several months, and those compounds showing promise for specificity and absence of toxicity will move to Task 4.

Task 3. ***Creation of iPSC-derived and transdifferentiated neurons from fibroblast lines***

Summary of current objectives – One of the main objectives of Task 3 was to isolate fibroblasts from human patients diagnosed with *Fmr1* full mutations to complement those that we already have developed and characterized in the premutation range (Liu et al., 2012); this task is essentially complete. A secondary objective was to establish a set of sensitive biomarkers that distinguish the morphological, signaling, and functional impairments that distinguish fragile X syndrome from premutation and normal neurons, as we had done previously in the mouse neuronal culture models. In this regard, we have observed strong correlations between the nature and degree of phenotypic divergence and the levels of FMRP expressed.

As noted above under Task 2, we have made promising observations with a fibroblast culture model, where we have identified a number of compounds that substantially increase FMRP levels. This finding makes the development of a neuronal culture model imperative so that the various functional readouts of the hippocampal neurons can be used to assess the effects of these compounds on a neuronal network model (Chen et al., 2010; Cao et al., 2013; Cao et al., 2012). Thus, we will shift our immediate objective to the assessment of the compounds (Task 2) using the mouse model, only later turning to the iPSC model for the most promising candidates.

Summary of Results - We had previously established that decreased FMRP is a consistent consequence of repeat expansion in the high-repeat range (170-200 CGG) in both human and mouse neuronal cells, as well as in the mouse premutation KI model (Ludwig et al., 2014).

We have more recently (unpublished) identified mechanistically linked biomarkers that are early etiological drivers of CGG-repeat-expansion–related neurological developmental impairments. The earliest of these is chronically elevated cytoplasmic Ca^{2+} and μ-calpain activation. We hypothesized that chronically activated μ-calpain would have a profound influence on the level of activated Cdk5 due to a prolonged shift in the balance of p35/p25, whose ratio tightly regulates the activity of Cdk5 during prenatal and postnatal development of the brain. Significantly, the activity of this multifunctional signaling kinase (Cdk5) is critical not only for proper synaptogenesis and neural network formation, but its unregulated activity is known to promote abnormal signaling to the nucleus and alter ATM phosphorylation that can influence temporal transcription patterns of key synaptic proteins. We do not know the

relationship between FMRP loss and these signaling abnormalities; however, they provide us with a range of outcome measures to test the effects of increasing FMRP.

Under the current Task, we have continued our investigation of key Ca^{2+} regulated pathways leading to abnormal activation of μ-calpain–CdK5–ATM signaling to the nucleus, which lead to mitochondrial oxidative stress and cellular Ca^{2+} overload. Here we have obtained new mechanistic data implicating this pathway in not only early impairments in morphological development, but also impaired glutamatergic neurotransmission. We have discovered consistent patterns of developmental dysfunction across the mouse *FMR1* KI mouse model and iPSC-derived neuronal precursor cells (NPCs) from *Fmr1* patients with either premutation or full mutation alleles.

Progress – We have observed that intracellular cytoplasmic Ca^{2+} is chronically elevated as early as 7 days in vitro, and that these Ca^{2+} levels appear to correlate with the level of FMRP deficiency, with human full mutation cells having the highest levels and greatest FMRP reduction. Importantly, elevated cytoplasmic Ca^{2+} and μ-calpain activity are coincident with reductions in the ratio of P35/P25, which relieves in inhibition of Cdk5. One major substrate of Cdk5 is nuclear ATM, and we observe that, as predicted, cortical tissues from brains of mice deficient in expression of FMRP (preCGG mice) have significantly higher levels of phosphorylated ATM (P-ATM) compared with wild type mice at the same age.

Upon closer examination of the distribution of ATM within neurons using confocal microscopy, we discovered a pattern of intranuclear puncta that are brighter and more abundant in FMRP-deficient neurons compared to wild type neurons (**Figure 4**).

Finally we have discovered evidence for abnormal glutamatergic neurotransmission that extends across mouse and human models, providing a functional biomarker associated with FMRP deficiency, a biomarker that lends itself to rapid throughput screening of small molecules that normalize FMRP levels (Task 2).

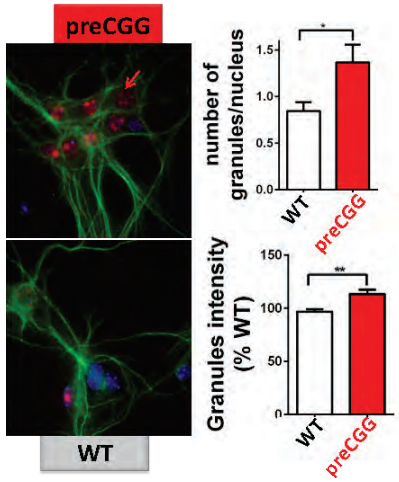

Figure 4. ATM puncta (red) in a premutation CGG-repeat (preCGG) allele, compared to a wild type (WT) allele, using confocal microscopy in mouse neurons.

Accomplishments/Discussion – Collectively concordant results from both our mouse and human models of FMRP deficiency provide an unprecedented opportunity to screen small molecule leads (increase in FMRP) identified under Task 2. We have identified morphological, biochemical, and functional biomarkers that not only are likely to make etiological contributions to the developmental impairments produced by FMRP deficiency, but also will facilitate targeted screening of small molecules that could ameliorate the onset or severity of these biomarkers.

Collectively, our experimental models suggest that they would be useful in defining common etiological mechanisms that promote early-onset developmental impairments in neuronal connectivity and later progression of neuropathological sequelae. Importantly, our mouse and human models underscore the translational potential of screening small molecule libraries that enhance expression of FMRP (described under Task 2), during the entire prenatal and postnatal developmental timeframe, for their therapeutic potential in normalizing morphometric, signaling, and synaptic functions in an iterative manner.

Task 4. *Extension of small molecule targeting of the FMR1 mRNA in premutation (preCGG) mouse and human iPSC and transdifferentiated neurons*.

Summary of current objectives – The primary objective of the entire project is the identification and further characterization of small molecules that are capable of increasing FMRP levels in neuronal cells (mouse or human iPSC-derived cells). The largely negative results of Task 1 (modest 1.8-fold increase in FMRP), coupled with the anticipated difficulties of delivery of oligomeric RNA molecules to the CNS, suggest that further effort under Task 1 would not be fruitful. Moreover, the exciting results under Task 2, with a number of compounds (all with predicted ability to cross the blood-brain-barrier) identified that are capable of increasing FMRP levels by up to 6-fold over normal, have allowed us to focus our efforts toward further studies with those compounds. The development of highly-sensitive outcome measures under Task 3 will allow us to proceed to Task 4, focused entirely on the "hit compounds" of Task 2. Thus, the principal objective under Task 4, while largely unchanged from the original proposal, is now more focused toward candidate small molecules.

Summary of Results/Progress – Since we have only recently identified suitable "hit" compounds for further study, we are only now beginning to carry out Task 4. We are currently about 12% of the way through our compound screen (Task 2), but already have a number of compounds (6 cpds with >3-fold increase in FMRP, max 6-fold increase; 6 cpds with 2–3-fold increase) that are appropriate for further analysis using the tools developed under Task 3. Thus, we feel it appropriate to start evaluating the compounds. The initial efforts will involve a broad characterization of how they might be causing the increase in FMRP: increased transcription, increased translation, gene-specific or general increase, etc. Such studies are very rapid, and will take place while the more time-intensive studies of neuronal function are carried out. Since we will continue to screen the remaining compound library over the next 5-7 months, we will likely have additional candidates to screen.

Once we have identified promising candidates, based in part on their ability to reverse the abnormal neuronal morphology and function, the next logical step would be to test these compounds in mice to (*i*) demonstrate the ability of the compounds to cross the blood-brain-barrier, and (*ii*) to determine whether there is any evident toxicity or functional improvement (**Table 1**). Given the time and expense of carrying out the necessary studies in mice, these efforts will naturally extend beyond the end of the current grant period and would be the basis for securing additional funding for that effort.

Table 1. Outcome measures for *in vitro* neuronal studies of "hit" compounds from Task 2.

Outcome domain	Outcome measure
Morphological	Neuronal complexity (Chen et al., 2010; Liu et al., 2012)
	Reduction of ATM and P-ATM within the nucleus
Biochemical	Normalization of μ-calpain activity
	Normalization of p35/p25
	Normalization of CdK5 activity
Functional	Normalization of cytoplasmic resting Ca^{2+}
	Normalization of abnormal glutamatergic signaling (Liu et al., 2012)
	Normalization of abnormal burst electrical firing of neuronal networks

4. KEY RESEARCH ACCOMPLISHMENTS

- We have implemented and validated a high-throughput, quantitative assay for FMRP levels. This assay (Cisbio, Inc) is based on the coordinated binding of two fluor-labeled antibodies to a single protein, with energy transfer between the two fluors (FRET) being a

measure of the total FMRP. We have now run over twelve thousand individual measurements using this assay. This assay has contributed to the major goals of this project (Tasks 1 and 2) to screen for compounds that increase FMRP levels. Beyond these goals, the assay will have a profound benefit as a rapid, quantitative measure of FMRP, itself directly related to clinical involvement/outcome.

- As an interim accomplishment, we have identified a number of small molecules that, upon single dose of the compound to patient fibroblasts in culture, have raised the level of FMRP by at least 5-fold within a 24 hr period. What is particularly gratifying is that we have found these compounds after screening only about 12% of our total library (2,400 compounds so far from the 20,000-cpd library) The implication of our findings are potentially huge: if additional studies bear out these initial findings, then there exists the potential for one or more of the compounds to eventually be used as a targeted therapeutic approach to fragile X syndrome, and perhaps more broadly to autism and to associated seizure activity.
- Under Task 3, we have developed a series of refined outcome measures in neuronal cells (mouse and human iPSC-derived neurons) to allow us to evaluate the potential therapeutic benefit of "hit" compounds identified under Task 2.

5. CONCLUSION

Our discovery of a small number of "hit" compounds under Task 2 is potentially extremely important for targeted treatment for fragile X syndrome, the leading single-gene form of intellectual disability and leading known form of autism. The "potential" nature of these findings reflects the need for follow-on studies to characterize the mechanism of action of the chemical compounds, their selectivity (increase only FMRP, or other proteins as well?), evidence of toxicity (we have not seen any evidence of reduced cell viability) in both cell and animal models, evidence for distribution to the brain, and finally, evidence of FMRP up-regulation in the animal model.

For the remainder of year three of the current project, we will focus on the further characterization of the current "hit" compounds, continue our search for additional hits, and will initiate further efforts (in both cell and animal models) to assess neuron-specific effects of these candidates (using our neuronal cell measures), CNS penetration, and potential toxicity. Studies of efficacy in the mouse model will necessarily be left for future studies, which we feel are important to carry out as soon as is practicable.

Fundamentally, we now have the potential for the development of a targeted therapeutic approach to the most common single-gene disorder giving rise to cognitive impairment and autism. Beyond this, elevation of FMRP would hold the potential for the treatment of both PTSD and one or more of the major psychiatric disorders, based on the observations (for the latter) that FMRP levels are decreased in the brains of individuals who suffered from major depression, bipolar disorder, and schizophrenia.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. *Manuscripts submitted for publication during the period covered by this report resulting from this project.*

1. Lay Press: N/A

2. Peer-Reviewed Scientific Journals:

Berman RF, Buijsen RA, Usdin K, Pintado E, Kooy F, Pretto D, Pessah IN, Nelson DL, Zalewski Z, Charlet-Bergeurand N, Willemsen R and Hukema RK (2014) Mouse models of the fragile X premutation and fragile X-associated tremor/ataxia syndrome. *J Neurodev Disord* 6:25. PMID25136376, PMC4135345

3. Invited Articles: N/A

4. ***Abstracts:*** N/A

b. ***Presentations made during the last year (international, national, local societies, military meetings, etc.).***

Gaëlle Robin, Jose R. López, Susan Hulsizer, Paul J. Hagerman, Isaac N. Pessah Ataxia-telangiectasia mutated (ATM) signaling is up-regulated in an *FMR1* premutation mouse model. Keystone Symposium- Pathways of Neurodevelopmental Disorders, March 17, 2015 Tahoe City, California, USA.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report

8. REPORTABLE OUTCOMES

Nothing to report

9. OTHER ACHIEVEMENTS

Nothing to report

10. REFERENCES

Cao Z, Hulsizer S, Cui Y, Pretto DL, Kim KH, Hagerman PJ, Tassone F and Pessah IN (2013) Enhanced asynchronous Ca^{2+} oscillations associated with impaired glutamate transport in cortical astrocytes expressing *FMR1* gene premutation expansion. *J Biol Chem* 288:13831-13841. PMID23553633, PMC3650419

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Schutzius G, Bleckmann D, Kapps-Fouthier S, di Giorgio F, Gerhartz B and Weiss A (2013) A quantitative homogeneous assay for fragile X mental retardation 1 protein. *J Neurodev Disord* 5:8. PMID23548045, PMC3635944

11. APPENDICES

Nothing added